J. Biochem. Mol. Biol. Vol. 30, No. 4, pp. 274~279 July 31, 1997

Purification and Characterization of Acetolactate Synthase from Barley

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(Received May 15, 1997)

Abstract : Acetolactate synthase (ALS) catalyzes the first common step in the biosynthesis of branched-chain amino acids, valine, leucine, and isoleucine. ALS is the target site for several structually diverse classes of herbicides including sulfonylureas, imidazolinones, and triazolopyrimidines. We have purified ALS from etiolated barley shoots to homogeneity. The five major purification steps are ammonium sulfate fractionation, DEAE anion exchange, hydroxylapatite, Bio-Gel A gel filtration, and low pressure Mono-Q chromatography. Approximately 170-fold purification was achieved and the yield was 0.45% of initial activity in the crude extract. Both SDS-PAGE and Western blot analysis showed a single polypeptide of ALS with an apparent molecular mass of 64 kDa. The result of nondenaturing gel electrophoresis with activity staining indicated that the molecular mass of its native form is approximately 225 to 250 kDa. The values of K_m for pyruvate, pI, and optimum pH of ALS were determined to be 2.0 mM, 5.2, and 7.0, respectively. Feedback inhibition studies showed that ALS is more susceptible to leucine than valine. And IC_{50} value of Cadre, a class of imidazolinones, is about 1.5 μM for ALS.

Key words : acetolactate synthase, barley, imidazolinone, purification

Acetolactate synthase (ALS, EC 4. 1. 3. 18; also referred to as acetohydroxyacid synthase) is the first common enzyme in the biosynthesis of valine, leucine, and isoleucine in plants and microorganisms. ALS can either catalyze the condensation of 2-acetolactate from two molecules of pyruvate in the first step of valine and leucine synthetic pathway, or the formation of 2-aceto-2-hydroxybutyrate from one molecule of pyruvate and 2-ketobutyrate as the second step of isoleucine biosynthesis. ALS has attracted a great deal of attention as the target site for several classes of structually diverse herbicides, including sulfonylureas (LaRossa and Schloss, 1984; Ray, 1984), imidazolinones (Shaner *et al.*, 1984), and triazolopyrimidines (Kleschick, 1984).

In *Escherichia coli* and *Salmonella typhimurium*, three ALS isozymes differing in substrate preference and feedback regulation have been identified and purified (Grimminger and Umbarger, 1979; Schloss *et al.*, 1985). The enzymes from these enterobacteria exist as tetramers composed of two large and two small subunits with apparent molecular mass of 60 kDa and 9 to 17 kDa, respectively (Grimminger and Umbarger, 1979; Schloss *et al.*, 1985). However, low abundance and extreme lability have hampered purification and biochemical characterization of the enzyme from plant sources. Thus little is known of plant enzymes about structure, subunits, specificity and herbicidal interaction. Recently the ALS gene from several sources has been cloned (Mazur and Falco, 1989; Singh *et al.*, 1991), and ALS from *Arabidopsis thaliana* and *Brassica napus* have been expressed in *E. coli* and *S. typhimurium*, respectively (Smith *et al.*, 1989; Wiersma *et al.*, 1990).

Previously, ALS from barley and pea was partially purified and characterized (Lee *et al.*, 1991). Inhibition studies were also carried out using various types of pyrimidine derivatives (a new class of ALS-inhibiting herbicide), sulfonylureas, and imidazolinones (Choi *et al.*, 1993; Shim *et al.*, 1995). In this work, we describe a procedure to purify ALS from barley to homogeneity and some physical and kinetic properties of the enzyme.

Materials and Methods

Materials

Barley seeds were sowed in the vermiculate. These shoots were grown in a dark room at 25°C for 6 days and then harvested. DEAE-Sephacel, hydroxylapatite, Mono-Q, Bio-Gel A and Heparin were purchased from Bio-Rad Laboratories (Hercules, USA). Sodium pyruvate, thiamine pyrophosphate (TPP), Sephadex G-25, and α-naphthol were from Sigma Chemical Co. (St. Louis,

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USA). Series of isoelectirc focusing agents were from NOVEX (St. Roselle, USA). Cadre was given by Dr. Dae-Whang Kim (Korea Research Institute of Chemical Technology, Taejon, Korea). Anti-ALS antibody and malE-ALS fusion protein were obtained from Plant Biotechnology Institute of National Research Council in Canada. Anti-ALS antibody is against malE-ALS fusion protein which is the protein expressed from *E. coli* transformed by pBI-684 (Bekkaoui *et al.*, 1993). *E. coli* strain, MF 2000, and pTATX (Smith *et al.*, 1989) were obtained from Dr. B. J. Mazur (Du Pont).

Enzyme assay

The assay mixture contained 20 mM potassium phosphate, pH 7.0, 0.5 mM TPP, 10 μM FAD, 20 mM pyruvate, 50 mM magnesium chloride plus enzyme in the final volume of 200 μl. After incubation at 35°C for 60 min, the reaction was stopped by the addition of 20 μl of 6 N H_2SO_4. The reaction product (acetolactate) was allowed to decarboxylate at 60°C for 15 min. The acetoin formed was determined according to Westerfeld (1943) by incubating with 200 μl of 0.5% (w/v) creatine and 200 μl of 5% α-naphthol at 60°C for 15 min. Then the absorbance of the mixture was measured at 525 nm. The concentration of protein was determined by the method of Lowry *et al.* (1951). Specific activity of ALS is defined as mole of acetoin/min/mg of protein. The IC_{50} value for inhibition is defined as the concentration of inhibitor which inhibits 50% of ALS activity.

Gel electrophoresis

SDS-PAGE was performed on 10% of polyacrylamide gel containing 0.1% of SDS. Nondenaturing gel electrophoresis was done according to the method of Laemmli (1970) without SDS in 7.5% acrylamide separating gel and 4% stacking gel.

Native isoelectric focusing was carried out on a 5% polyacrylamide gel in which the pH gradient was 3 to 10. The gel contained 2% ampholytes without denaturing reagents, and focusing conditions were adapted from Robertson *et al.* (1987).

Activity staining

Activity staining was done as described by Grimminger and Umbarger (1970). After the nondenaturing gel electrophoresis, the gel was sliced into 1.5 mm pieces and incubated in the assay mixture for 1 to 3 hr at 37°C and stained with creatine and α-naphthol as described in the enzyme assay.

Western blot analysis

The samples were electrophoresed on the gel with 0.1 % SDS and transfered to nitrocellulose membrane. Hybridization was carried out with first antibody (*mal*E-ALS antibody) for 6 h shaking incubation and then blots were washed with blocking solution. On this transferred membrane, second antibody-labelled HRP (Horseradish peroxidase) was hybridized. The membrane sheets were washed with 20 mM Tris, pH 7.5, 0.5 M NaCl (TBS) containing 0.05% Tween-20 and detected with ECL method described in the manufactor's protocol (Amersham corporation, Arlington Heights, USA)

Purification of barley acetolactate synthase

All operations were performed at 4°C and minimized light exposure during homogenization, chromatography, and other experiments. Usually 180 g barley shoots frozen by liquid nitrogen were homogenized in a volume of 800 ml standard buffer (50 mM Tris-HCl, pH 7.8, 15% glycerol, 1 mM EDTA, 1 mM DTT) containing 5 mM L-leucine, 50 μM FAD, and 5 mM $MgCl_2$. The shoots were homogenized in a blender for 200 second period (5 times ×40 seconds). Homogenate was centrifuged for 5 min at 20,000 g. Solid ammonium sulfate was added to the supernatant (30% saturation, 1.76 g/10 ml) and the precipitate was removed by centrifugation for 20 min at 24,000 g. The supernatant was treated to 45% saturation of ammonium sulfate fractionation, and the sample was centrifuged for 20 min at 24,000 g. The precipitate was dissolved in a minimum volume of the standard buffer and desalted on Sephadex G-25 column (2.5×18 cm). The desalted sample was applied to DEAE-Sephacel column (2.8×12 cm) equilibrated with standard buffer containing 1 mM DTT. The column was washed with the same buffer until the absorbance of eluent at 280 nm was near 0, and then the bound proteins were eluted with a linear gradient of 0 to 300 mM NaCl. Active fractions were pooled and concentrated by ultrafiltration using Amicon-Cell with a PM-30 membrane. The concentrated proteins were loaded on hydroxylapatite (2.8×10 cm; Bio-Gel HTP). The column was washed with 25 mM potassium phosphate buffer containing 15% glycerol, 1 mM DTT, 5 mM $MgCl_2$, and 5 μM $CaCl_2$ until all unbound proteins were removed, and then eluted with a potassium phosphate gradient of 25 to 250 mM. Fractions containing ALS activity were pooled and concentrated by ultrafiltration using Amicon-Cell with PM-30 membrane. Subsequently, the proteins were applied on Bio-Gel A gel filtration (2.2×45 cm) equilibrated with standard buffer containing 5 mM pyruvate, and 5 mM L-leucine. Active fractions were pooled and concentrated by Amicon Centricon-30. The protein solution was loaded on a strong anion exchange Mono-Q column equilibrated with standard buffer and the bound proteins were eluted with a NaCl gradient of 0 to 300 mM. Active frac-

Table 1. Purification of acetolactate synthase from 180 g etiolated barley shoots

Procedure	Activity (U[a])	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Homogenate	59.07	3938.0	0.015	100.0	1
30-45% $(NH_4)_2SO_4$	25.20	126.75	0.20	40.0	12
DEAE-Sephacel	9.20	10.35	0.89	15.0	56
Hydroxylapatite	2.43	1.37	1.77	4.0	112
Gel filtration	0.71	0.30	2.38	1.2	151
Mono-Q	0.28	0.105	2.67	0.45	169

[a]One unit (U) was defined as the μmol of synthesized acetolactate per minute.

tions were concentrated by Ultrafree MC filter-10 and stored in liquid nitrogen.

Results

Purification of barley acetolactate synthase

The purification of ALS from barley shoots to homogeneity was carried out using a procedure which has five major steps, ammonium sulfate fractionation, DEAE anion-exchange, hydroxylapitite, Bio-Gel A gel filtration, and low pressure Mono-Q chromatography. The results of an average purification are presented in Table 1. Approximately 170-fold purification was achieved, but the overall yield was only 0.45% of initial activity in crude extract. The specific activity eventually obtained was 2.67 μmol min^{-1} mg^{-1}. The value is significantly higher than the value of 1.59 μmol min^{-1} mg^{-1} reported by Durner and Böger (1988). They have purified ALS from barley shoot to near homogeneity. In this purification only about 100 μg of pure ALS could be obtained from 180 g etiolated barley shoot. Figs. 1A, 1B, and 1C show the elution profiles of DEAE-Sephacel, hydroxylapatite, and Bio-Gel A gel filtration, respectively. Each step gave a good separation of ALS from other proteins, and the performance of each step was reasonably reproducible. But a large portion of ALS activity was lost at each separation step.

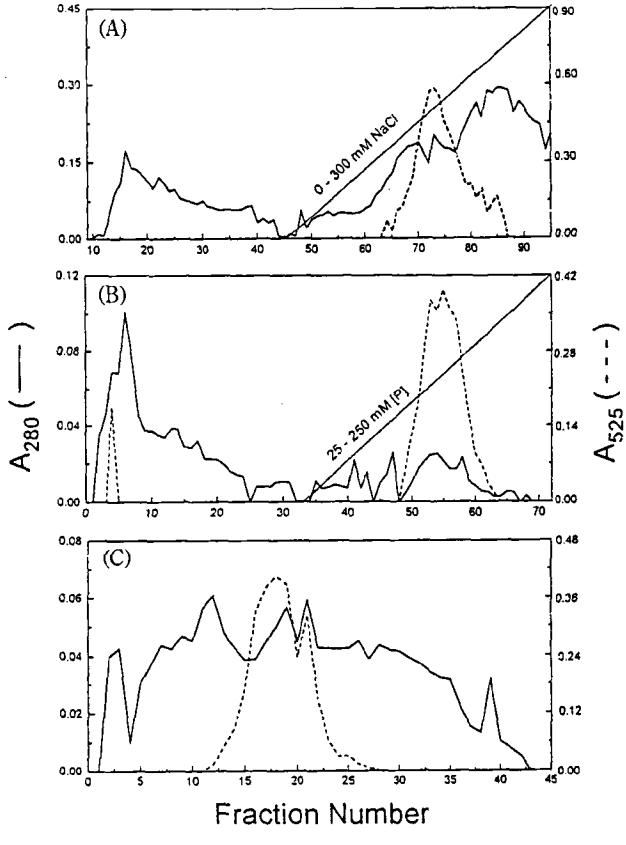

Fig. 1. (A) The elution profile of barley acetolactate synthase from DEAE-Sephacel column. Elution was performed by a linear NaCl gradient from 0 to 300 mM. Protein, —; Enzyme activity, ---. (B) The elution profile of barley acetolactate synthase from hydroxylapatite. Elution was performed by a linear phosphate gradient from 25 to 250 mM. Protein, —; Enzyme activity, ---. (C) The elution profile of barley acetolactate synthase from gel filtration. Protein, —; Enzyme activity, ---.

Characterization of barley acetolactate synthase

The protein pattern resolved by SDS-gel electrophoresis for samples at the last two stages of purification are shown in Fig. 2. The last purification step on Mono-Q chromatography led to 64 kDa band of ALS subunit.

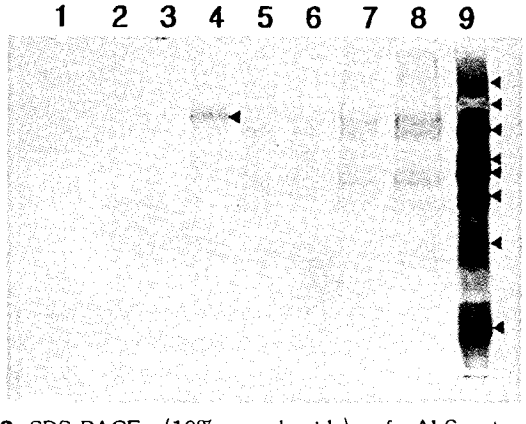

Fig. 2. SDS-PAGE (10% acrylamide) of ALS at chromatography of Mono-Q column. The gel was stained with silver nitrate. Lanes 1-7, eluted fractions of Mono-Q chromatography; lane 8, sample of gel filtration; lane 9, molecular markers, 97.4, 66.2, 55.0, 42.7, 40.0, 31.0, 21.5, 14.3 kDa.

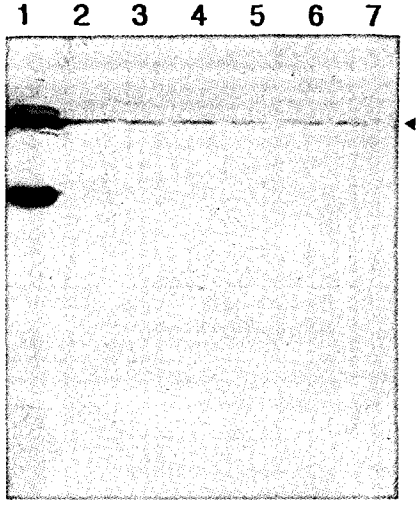

Fig. 3. Western blot analysis using anti-ALS antibody. After SDS-PAGE, proteins were transferred onto a nitrocellulose membrane and detected by ECL according to the procedures described in "Materials and Methods". Lane 1, cell extracts expressed by pTATX (vector containing ALS gene, Smith *et al.*, 1989); lanes 2-3, gel filtration (lane 2: concentrated with Centricon-30); lane 4, hydroxylapatite; lane 5, DEAE-Sephacel; lane 6, ammonium sulfate fractionation desalted with Sephadex G-25; lane 7, homogenate.

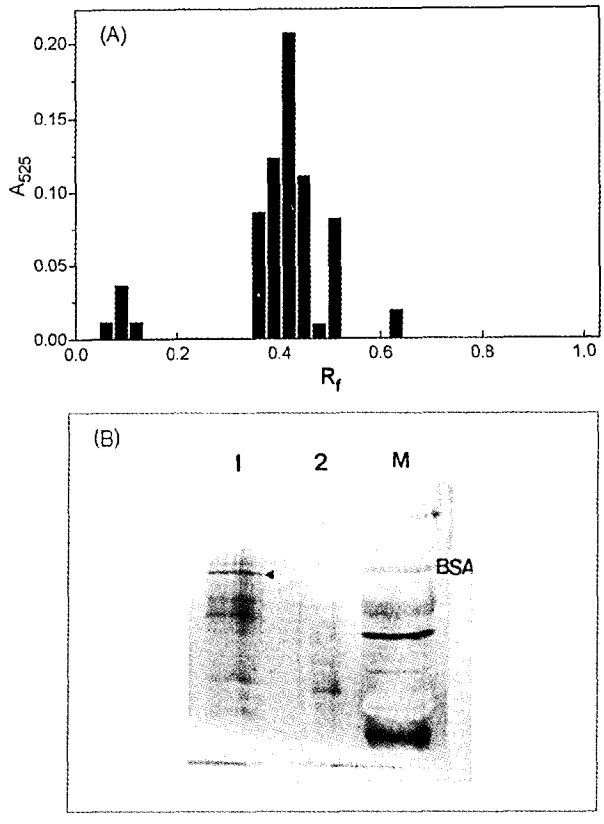

Fig. 4. (A) Activity staining profile of barley ALS after nondenaturing gel electrophoresis of hydroxylapatite. The gel was sliced into 1.5 mm pieces and measured for the activity of ALS. (B) 2-demensional SDS-PAGE of ALS active gel slice after activity stainig. Lane 1, ALS active gel slice; lane 2, negative; lane 3, markers.

The result was confirmed by Western blot analysis of samples of each step using polyclonal anti-ALS antibody (Fig. 3). The samples from each step showed the same band as shown by purified ALS after Mono-Q chromatography. The second band of lane 1 was probably maltose binding proteins or other proteins nonspecifically cross-reacted with polyclonal antibodies of *malE*-ALS2 fusion protein (Bekkaoui *et al.*, 1993).

The molecular mass of active form of barley ALS was determined to be approximately 225 to 250 kDa by nondenaturing gel electrophoresis followed activity staining (Fig. 4A). Two dimensional SDS-PAGE of ALS active slice gel showed 64 kDa ALS subunit (Fig. 4B). This result implies that active form of ALS is likely tetrameric oligomer.

The pI value of native barley ALS was determined by isoelectric focusing followed activity assay to be 5.2 (Fig. 5). This indicates that the active form ALS is somewhat acidic. The study of pH-dependent ALS activity of active fractions through Bio-Gel A step over pH range from 4.0 to 10.0 showed maximum activity at pH 7.0 (data not shown). The kinetic curve of ALS with respect to pyruvate showed a very slight deviation from Michaelis-Menten hyperbolic pattern and maximum activity was reached at approximately 45 mM pyruvate. But Lineweaver-Burk plot showed no significant deviation from a straight line and gave the K_m value of 3.0 mM (Fig. 6).

The effect of branched-chain amino acids on the ALS activity is shown in Fig. 7A. Inhibition of ALS was observed with leucine and to a lesser extent with valine. Isoleucine also inhibited ALS but very slightly at mil-

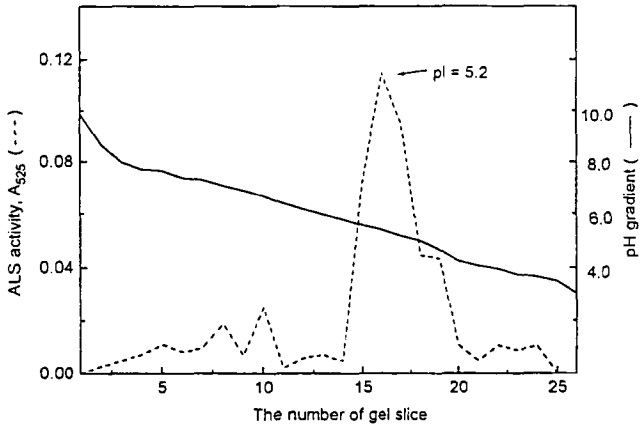

Fig. 5. Native isoelectric focusing. The loaded sample was through Bio-Gel A gel filtration. The pH gradient was 3 to 10 on the 5% polyacrylamide gel without denaturing agents. The site on the gel having the highest ALS activity was pH 5.2. pH gradient, —; Enzyme activity, ---.

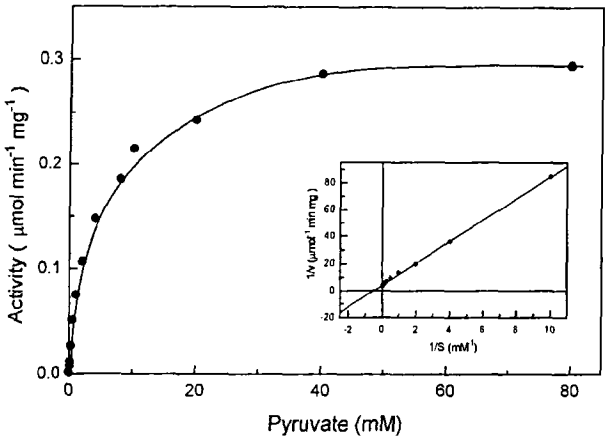

Fig. 6. Substrate saturation curve for pyruvate with acetolactate synthase The ALS purified from Bio-Gel A gel filtration was assayed under the standard condition reaction as described in "Materials and Methods". The insert is Lineweaver-Burk plot of the data.

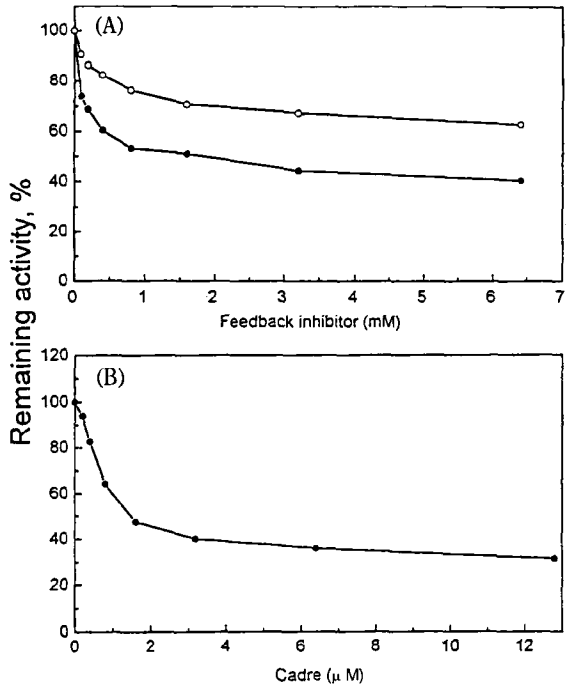

Fig. 7. (A) Feedback inhibition of barley acetolactate synthase by leucine and valine. Leucine, ●; Valine, ▲. (B) Inhibition of barley acetolactate synthase by Cadre, a class of imidizolinone.

limolar level (data not shown). ALS inhibiting commercial herbicide imidazolinone Cadre inhibited barley ALS effectively with IC_{50} value of 1.5 μM (Fig. 7B).

Discussion

Acetolactate synthase plays an important role in the regulation of biosynthesis of amino acids, valine, leucine, and isoleucine, since the enzyme is subject to feedback inhibition by these end products. ALS is a unique herbicide target in that structurally diverse classes of herbicides inhibit the enzyme. ALS is one of the most prominent and attractive targets of herbicides since the level of ALS in plants is very low and its inhibition by herbicides simultaneously blocks the biosynthesis of three essential amino acids, valine, leucine, and isoleucine. To design new and potent herbicides, the structural information of plant ALS is absolutely necessary. A great deal of effort has been devoted to purify ALS from plant sources. However, the purification of plant ALS to homogeneity has been hampered by its low abundance and extreme lability. Thus, the studies of subunit, structure, and reaction mechanism of plant ALS have not been made with pure ALS. In this work, ALS was initially purified from barley shoot to homogeneity by the criteria of SDS-PAGE. But the overall yield was exceptionally low. While loss of activity after ammonium sulfate fractionation in the presence of 50 μM FAD and 15% glycerol was less than 20% when stored at 70 K, purification was greatly hampered by an extreme lability during the chromatographic steps. At each chromatographic step more than 60% of ALS activity was lost.

The last purification step on Mono-Q chromatography led to a single 64 kDa band SDS-PAGE (Fig. 2). Singh *et al.* (1991) reported a similar result in which the molecular mass of barley ALS in crude extract was 65 kDa determined by Western blot analysis using polyclonal antibody against *Arabidopsis* ALS. Duner and Böger (1988), however, reported that the molecular mass of a single polypeptide of barley ALS was 58 kDa.

Recently, Chang *et al.* (1997) detected both 64 and 58 kDa polypeptides of barley ALS by Western blot analysis using polycolonal antibody against tobacco ALS. But 58 kDa ALS subunit was detected only after storage of 64 kDa ALS at -20°C for several days. It seems that 58 kDa ALS subunit is likely a degraded product from 64 kDa ALS polypeptide.

The molecular mass of native barley ALS as de- termined by nondenaturing gel electrophoresis was approximately 225 kDa to 250 kDa. This result indicates that native barley ALS is a tetramer of identical subunits. Reports on bacterial ALS (Grimminger and Umbarger, 1979; Eoyang and Silverman, 1984; Schloss *et al.*, 1985) have shown that the enzyme is composed of two large (59-60 kDa) and two small (9-17 kDa) subunits. Yeast ALS was reported to be a dimer composed of two subunits, each with molecular mass of 75 kDa (Poulsen and Stougaard, 1989).

We found a pH optimum for barley ALS at pH 7.0 (Data not shown). This is rather high compared to the

result by Duner and Böger (1988) who found a broad pH optimum around 6.5 for ALS in crude extract of barley shoots. The K_m of 3 mM for pyruvate found here agrees reasonably well with the K_m of 5 mM for pyruvate determined by Duner and Böger (1988).

Inhibition of ALS activity by imidazolinone herbicide Cadre (Fig. 7B) suggests that the apparent ALS activity came from ALS and not from pyruvate decarboxylase. Pyruvate decarboxylase can also form acetoin directly from two molecules of pyruvate, but in contrast to ALS, pyruvate decarboxylase is not inhibited by imidazolinone herbicide (Muhitch *et al.*, 1987).

Acknowledgements

This work was supported by research grants from the Korea Science and Engineering Foundation (92-4600-01-01-3/95-0402-09-01-3) and the Basic Science Research Instituite Program from Ministry of Education, the Republic of Korea (BSRI-95-3434).

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